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**MODULATING RADIATION RESISTANCE: Novel
Protection Paradigms Based on Defenses against Ionizing
Radiation in the Extremophile *Deinococcus radiodurans***

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Uniformed Services University of the Health Sciences

**JULY 2013
Final Report**

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**AIR FORCE RESEARCH LABORATORY
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- **Date:** May 30, 2013
- **Title:** Modulating Radiation Resistance: Novel Protection Paradigms Based on Defenses against Ionizing Radiation in the Extremophile *Deinococcus radiodurans*
- **Project: AFOSR Award:** FA9550-10-1-0118. Funded: April 15, 2010
- **Present Status:** Closed
- **Technical Topic Area: 'Extremophile Initiative' - AFOSR Science Elements Addressed:** 1) *Discover the mechanisms for survival in extremophiles*; and 2) *Explore methods for exporting these protective strategies outside of the host cell*.
- **First Annual Report:** Submitted by M. J. Daly on April 3, 2011.
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Third/Final Report: Submitted by M. J. Daly on May 30, 2013.
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- **Program Financial Management:** Henry M. Jackson Foundation for the Advancement of Military Medicine • **HMJF Contact:** Mai Bui (mbui@hjf.org) Grants Manager, OSP, The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. 1401 Rockville Pike, Suite 600, Rockville, MD 20852.

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2. Original Objectives

The two objectives listed in Daly's 2009 AFOSR application:

(1) Develop novel radioprotectors and antioxidants based on catalytic Mn(II,III) redox-cycling processes identified in the extremely radiation-resistant bacterium *Deinococcus radiodurans*; and (2) Develop quantitative assays for oxidative protein damage as indicators of radiation exposure in prokaryotes (bacteria) and eukaryotes (yeast and fungi), and towards investigating the molecular mechanisms underlying Mn-dependent antioxidant processes *in vivo*. Progress towards these goals has presented the DoD and the greater scientific community with a novel and highly defensive chemical strategy to combat oxidative stress in diverse areas, including bioremediation, long-term enzyme storage, towards pre-exposure and post-exposure interventions against ionizing radiation in humans, and developing protective vaccines against emerging or existing pathogens and their WMD counterparts.

Following the *Annual AFOSR Performance Review* (Jan. 7-11, 2013), Dr. De Long advised Dr. Daly to continue to focus on characterizing the mechanisms of radioprotection of *D. radiodurans* complexes, particularly using advanced paramagnetic spectroscopy approaches in collaboration with Dr. Brian Hoffman.

3. Summary of 2012-2013 Effort (150 words):

In 2012-2013, we demonstrated that: **1)** Mn²⁺ complexes in living *D. radiodurans* cells are distinctly different from those in radiosensitive cells. As demonstrated by ENDOR/EPR/ESEEM spectroscopy, Mn²⁺ in *D. radiodurans* is bound mainly to nitrogenous ligands, forming complexes with orthophosphate (Pi) which are extremely resistant to gamma-radiation and preserve the *D. radiodurans* proteome. This work was published in *PNAS USA* (2013); **2)** Many environmental yeast are extremely radiation-resistant, accumulate nitrogenous Mn²⁺-Pi complexes, and highly resistant to radiation-induced protein oxidation; **3)** The radioprotective nature of Mn-peptide complexes is more dependent on the amino acid composition than the order of amino acids. A variety of decapeptide sequences were screened for their ability to protect protein structure and function following exposure to megadoses of ionizing radiation; and **4)** The Mn-peptide-Pi vaccine approach developed for methicillin-resistant *Staphylococcus aureus* (MRSA), published in *Cell Host & Microbe* (2012), was successfully applied to Venezuelan equine encephalitis virus.

4. Status of Work: Progress for Period April 2010-April 2013:

Background: The bacterium *D. radiodurans* represents life's utmost limit for ionizing radiation resistance. In spite of intensive efforts, the goal of exporting the protective processes of *D. radiodurans* outside of the host cell for practical purposes has eluded researchers for decades. The critical contribution of intracellular Mn complexes in defending *Deinococcus* cells from oxidative damage following exposure to x-rays and γ -rays, ultraviolet (UV) radiation, desiccation and redox-active toxins was first reported by Daly's group. That body of research was funded by AFOSR and now has become the foundation of a new field based on the concept 'Death by Protein Damage.' Since then, the critical role of intracellular small-molecule antioxidants in protecting proteins has been established in various model organisms including bacterial spores, vegetative bacteria, archaea, and simple animals (*e.g.*, Krisko et al., 2012, www.pnas.org/cgi/doi/10.1073/pnas.1119762109). This progress holds theoretical and practical implications of the highest order, including bioremediation of high-level radioactive waste sites, and metabolic interventions at the cellular level which mitigate oxidative stress during irradiation

and aging. Another tangible application of Daly's research is the preparation of radiation-sterilized whole-bacterial cell, whole-virus, and protein vaccines without loss in immunogenicity. Applying *Deinococcus* Mn complexes towards vaccine development is arguably the most important product of the project as it promises to expedite the development of defenses against bioterror threats or emerging infections caused by poorly characterized new or rapidly mutating agents, such as pandemic influenza and HIV.

See below for 2012-2013 Publications: Project Products.

4.1 The Central Hypothesis which Drove the Project – Mn^{2+} complexes govern the functionality and efficiency of DNA repair in extremely radiation-resistant organisms. Thus, a better understanding of the mechanisms underlying Mn^{2+} metabolite-based protein protection in radiation-resistant cells could lead to better approaches to harness their activities in diverse settings. As very little is known about the solution structures and modes of action of cellular Mn antioxidants, we interrogated Mn-facilitated radiation resistance *in vivo* and *in vitro* using two complementary approaches: 1) Applied biochemical and advanced spectroscopic tools to characterize Mn^{2+} -metabolite complexes as they exist in *D. radiodurans* cells, and other prokaryotes and yeast; and 2) Applied quantitative radiochemical assays to characterize the mechanisms of ROS-scavenging by the Mn^{2+} complexes.

4.2 Mn Complexes in *Deinococcus*

Interspecies comparisons of irradiated bacteria, archaea, and simple eukaryotes show that the levels of protein damage are not only quantitatively related to the efficiency of DNA double strand break (DSB) repair and survival, but are mechanistically linked to the accumulation of Mn^{2+} . Notably, extreme radiation resistance in prokaryotes, and associated protein protection, is not dependent on the presence of antioxidant enzymes. Proteins in *Deinococcus radiodurans* are not inherently radiation resistant – *D. radiodurans* proteins lose their resistance when the cells are grown under conditions which limit Mn^{2+} uptake or prevent Mn^{2+} redox-cycling, and when the proteins are extracted. In contrast, proteins in naturally sensitive bacteria are as susceptible to oxidation as when they are purified. Ensuing studies showed that protein-free cell extracts of *D. radiodurans* and *Halobacterium salinarum* are armed with low-molecular-weight (LMW) reactive oxygen species (ROS)-scavenging Mn^{2+} complexes which include peptides bound to Mn^{2+} and Pi. Mn^{2+} and Pi form complexes which catalytically remove superoxide ($\text{O}_2^{\bullet-}$) via a disproportionation mechanism; and amino acids and peptides, which scavenge hydroxyl radicals (OH^{\bullet}) very efficiently, form complexes with Mn^{2+} which catalytically decompose hydrogen peroxide (H_2O_2). When reconstituted *in vitro* at physiologically relevant concentrations, these constituents interacted synergistically in preventing the inactivation of enzymes during high-dose irradiation. For example, at 50,000 Gy, Mn^{2+} -peptide-Pi complexes preserved 50% activity of the dodecameric enzyme glutamine synthetase (466 kDa), which is normally inactivated by 150 Gy; however, Mn-peptide-Pi did not significantly protect DNA from DSBs. Evidently, the quaternary structures of proteins and their functions can be preserved in aqueous solution by Mn^{2+} -metabolite complexes at doses of ionizing radiation which destroy similarly treated DNA. In summary, the action of Mn^{2+} in protecting cytosolic proteins from ROS appears to occur at two levels: (i) by replacing Fe^{2+} and other divalent cations (e.g., Mg^{2+} and Cu^{2+}) with Mn^{2+} as mononuclear cofactors in enzymes, active sites are protected from oxidative damage; and (ii) surplus Mn^{2+} (i.e., the portion of a cell's Mn^{2+} budget which is not bound to proteins) forms ROS-scavenging complexes with various metabolites which provide global protein protection and preserve the quaternary structures of irradiated enzymes. It is important to note, based on *in vitro* enzyme studies, that high (not extreme) levels of radiation resistance are predicted to occur in cells which accumulate metabolites without Mn^{2+} . Mn^{2+} accumulation is not a singular determinant of radiation resistance. Rather, Mn^{2+} boosts protein protection in cells by interacting synergistically with the pool of small-molecule metabolites built up in cells, which is particularly

important in aerobic environments. Numerous organisms which accumulate “compatible solutes” fit this model, including representative archaea, cyanobacteria, lichens, alpine yeast, and tardigrades.

4.3 Knowns and Unknowns of *Deinococcus* Mn²⁺ Complexes

It is worth reminding the reader that just six years ago, the ideas which drove this body of research were considered heretical. For fifty years, the central dogma of radiation biology had been that DSBs were the universal critical lesions in irradiated cells. We have demonstrated that old idea to be false using bacteria as models - the critical “target” molecules in most irradiated cells are proteins. Since our original reports, this conclusion has been reinforced by research on irradiated archaea by Dr. Jocelyne DiRuggiero’s group at Johns Hopkins University; on irradiated bacteria by Dr. Miroslav Radman’s group at the University of Paris; and on irradiated simple animals by Dr. Matthew Meselson’s group at Harvard University.

How proteins in radiation-resistant cells are protected from oxidation during irradiation has become the central focus of such research. Before 2007, the only known physiologic/genetic traits which distinguished extremely resistant cells from naturally sensitive cells were (i) Mn²⁺ accumulated in bacteria somehow facilitates resistance, and (ii) Mn²⁺ superoxide dismutase (SodA) is dispensable. Since 2010, we have reported Mn-dependent mechanisms of radiation resistance with the following conclusions:

- Mn²⁺ protects proteins, but not DNA from long-lived radiation-induced ROS.
- Mn²⁺ promotes the export of H₂O₂ from irradiated cells.
- Mn²⁺ redox-cycling in cells, and *in vitro*, scavenges superoxide, yielding H₂O₂.
- Mn²⁺ redox-cycling is knocked out by Fe²⁺/Fe³⁺ during irradiation.
- Mn²⁺ redox-cycling in cells is blocked by high pH, when protons are lacking.
- Mn²⁺ is the critical form of intracellular Mn in *Deinococcus*, not Mn³⁺ or Mn⁴⁺.
- Mn²⁺ protection is lost when substituted by Mg²⁺, Ca²⁺, Fe²⁺, Ni²⁺, Cu²⁺ or Zn²⁺.
- Mn²⁺ is strongly associated with Pi in bacterial cells.
- Mn²⁺ forms complexes with metabolites accumulated in bacteria.
- Mn²⁺ complex formation is induced by radiation in *D. radiodurans*.
- Mn²⁺-metabolite complexes include Mn-Pi, Mn-peptide-Pi, and Mn-nucleosides.
- Mn²⁺-peptide-Pi complexes are massively protective of proteins, not DNA.
- Mn²⁺-peptide complexes are not universally radioprotective of proteins.
- Mn²⁺-peptide radioprotection is dependent on amino acid composition.
- Mn²⁺-peptide complexes are most protective if they contain histidine and/or methionine.
- Mn²⁺ complexes of *D. radiodurans* and *Bacillus* spores form spontaneously.
- Mn²⁺ complexes in bacteria and yeast appear to share structural features.
- Mn²⁺ complexes with organics containing two C=O separated by one (N3)H.
- Mn²⁺ optimally supports ROS-scavenging *in vivo*, as free ions at ~200 μM.
- Mn²⁺ catalytically scavenges radiolytic superoxide *in vitro* at 1-10 mM.
- Mn²⁺ forms Mn³⁺/Mn⁴⁺ if Mn²⁺ is present *in vitro* at >100 mM during irradiation.

4.4 Harnessing Radioprotective Mn-Complexes of Extremophiles

The ability of bacterial Mn²⁺ complexes to protect cells and biomaterials from mega-doses of gamma radiation has been published, and is summarized below.

- *D. radiodurans* Mn²⁺ complexes confer on the radiation-sensitive wild-type bacterium *Escherichia coli* the ability to grow luxuriantly under high-level chronic γ-radiation. This holds the prospect of bioremediation (cleanup) of radioactive waste sites with myriad *E. coli* strains

which were previously engineered for toxic organic/heavy metal-decontamination in non-radioactive environments.

- *D. radiodurans* Mn^{2+} complexes confer on purified enzymes the ability to survive extended desiccation. This holds the prospect of long-term storage of dried preparations of enzymes, antibodies, and proteins used in fuel cells. This is important in the context of DoD field operations where refrigeration is not possible.
- *Bacillus* spore Mn^{2+} complexes confer on aqueous preparations of purified enzymes the ability to survive extreme acute irradiation. This holds the prospect of enzymatic decontamination of hydrated radioactive environments.
- *D. radiodurans* Mn^{2+} complexes confer on cultured human cells the ability to survive extreme cellular insults caused by ionizing radiation. Treatment of human Jurkat T cells with native *D. radiodurans* Mn^{2+} complexes rescued them from γ -ray exposures (16 Gy) which caused 560 DSBs per diploid cell; and a reconstituted *D. radiodurans* Mn^{2+} -peptide-Pi complex conferred on Jurkat T cells the ability to survive 100 Gy [Lamkin, T., Pangburn, H. *et al.*, AFOSR, unpublished]. These radiation survival levels are record-breaking. Thus, *D. radiodurans* Mn^{2+} complexes stand poised to help expand radiation countermeasures in diverse settings, from pre-exposure prophylactic interventions to post-exposure therapeutics.
- A reconstituted *D. radiodurans* Mn-peptide-Pi complex now forms the basis of a powerful new irradiated vaccine approach. Viruses and bacteria incubated in Mn^{2+} -peptide-Pi and exposed to 40,000 Gy were killed, but their lifeless “shells” retained the capacity to generate protective neutralizing antibodies in animals (**Fig. 1**). Without any risk of infection, the vaccine approach was successfully tested at NIH in mice against methicillin-resistant *Staphylococcus aureus* (MRSA), which kills about 18,000 Americans each year. This new strategy for vaccination could be applied to many other deadly diseases for which protective vaccines do not yet exist, including cholera, Ebola and HIV, and BW infectious agents (**Fig. 1**).

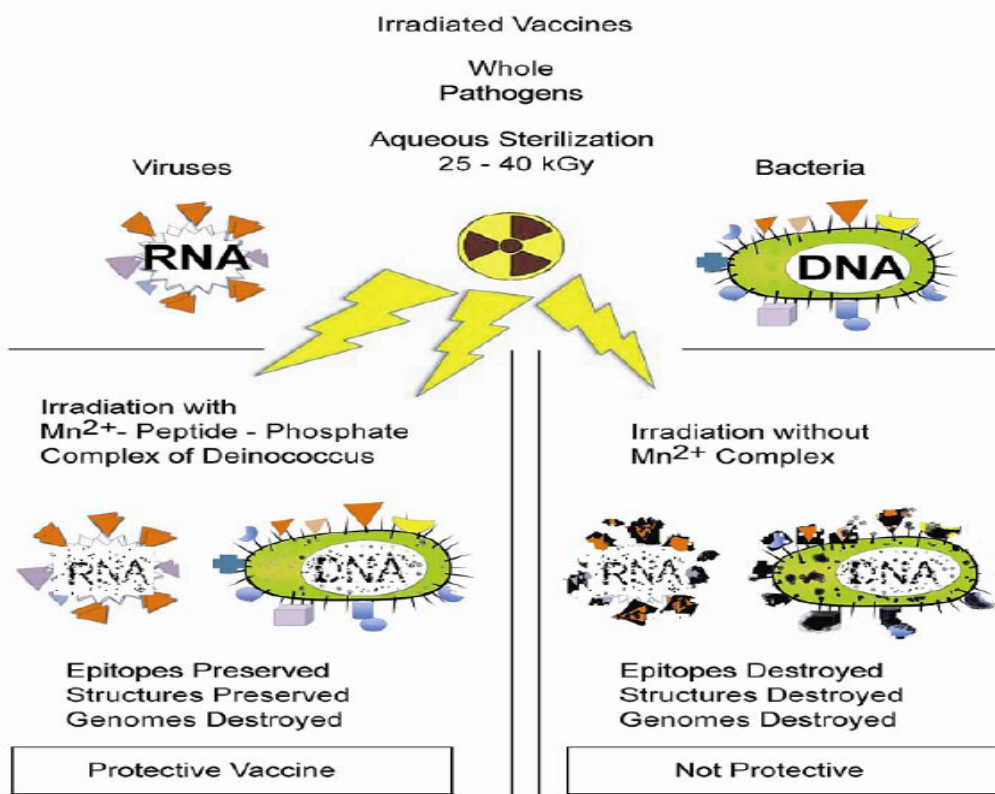


Figure 1. Graphical Abstract of Irradiated Vaccine Paper: A Mn^{2+} -Peptide-Phosphate complex of *Deinococcus radiodurans* preserves immunogenicity of lethally irradiated vaccines against viruses and bacteria (Gaidmakova *et al.*, 2012, see below).

Without knowing much about the mechanisms, we demonstrated that reconstituted *D. radiodurans* Mn^{2+} complexes potentially have powerful applications. Those transformational studies were reviewed by Daly in 2009 and 2012, and are strong arguments to continue this line of research if the AFOSR extremophile program is restored; the AFOSR Extremophile Initiative, established by MAJ Jennifer Gresham, was defunded following sequestration in Spring 2013.

4.5 Termination of AFOSR Extremophile Program following Budget Control Act of 2011 (Sequestration)

From Dr. Hugh C. De Long, DR-IV, DAF (4/13): “If you were up for renewal and had a grant in the system but it hasn't been awarded yet then you are indeed effected. Since the cut was significant, ALL grants of all programs that were not awarded WERE cut. All AFRL LRIR grants that were not fully funded yet will lose funding as well in their TD allotment. All increments that were not awarded may or may not have been cut depending on what your expenditures for 2012 were. As an example if you were owed an increment in 2013 and you had all of your 2012 money unspent then you won't be receiving your 2013 increment, and if you spent half of your 2012 money then you may only get half of your 2013 increment. There were two types of cuts. First was the swept funds which effects most of the renewals and late increments. The second was the science areas that we drop as the cut size grows. All of the extremophile program fell into this second category. That means different things to different people when it comes to FY14 (starting in Oct 2013). For FY14: If you had a grant in for renewal, I plan on resubmitting it (except extremophiles) for FY14 but this means there will be a gap in funding for effort. If you had an increment due this year and you receive an adjustment to its size then your grant will have to be renegotiated since in FY14 the plan currently is to give you your FY14 increment but the missing FY13 increment will not be made up in FY14. If you had a BRI grant that was awarded in summer last year, you will probably be effected by the increment adjustment. ALL of the extremophile program was cut. That means that unless I receive money back specifically for this program in FY14, it will be eliminated. I will have Pat Bradshaw keep those proposals around just in case it is resurrected. Even if the program returned next year there will be a gap in funding for them as well. Sorry this had to happen this year. Hopefully, it won't happen again next year, but the possibility does exist. It will all depend on where our budget this year ends up and what budget gets past for next year. I will update you further if there are any additional changes for both this year and next.”

4.6 Overview of Formal 2012 Grant Application submitted by Daly to AFOSR 12/10/12: Mn^{2+} Complexes: Interrogating the Mechanisms of Extreme Radiation Resistance in Extremophiles

Anonymous External Review of 2012 Daly Proposal:

Proposal Review Sheet

(Use additional sheets as needed & indicate the proposal number on each sheet)

Proposal Title: *Mn^{2+} Complexes: Interrogating the Mechanisms of Extreme Radiation Resistance in Extremophiles*

Principal Investigator: *Michael Daly PhD*

AFOSR Proposal Number:

Please rate your familiarity with the technical area represented by the proposal (choose from list using the check box)

- ☒ 1 – Very familiar – have worked in directly in the area
- ☐ 2 – Intermediate between 1 & 3
- ☐ 3 – Generally familiar – have limited experience in this area, or have worked in related areas
- ☐ 4 – Intermediate between 3 & 5
- ☐ 5 – General knowledge of the broad field, but not this specific area

Please rate your overall assessment of the proposal (choose from list using the check box)

- ☒ 7 – Reserved for a proposal of exceptional merit in terms of methodology, potential contribution to the state-of-the-art, and likelihood of producing results. One every few years (top 1%)
- ☐ 6 – Outstanding proposal of high technical merit, major contribution to the state-of-the-art, excellent approach likely to produce expected results. A few each year (top 3-5%)
- ☐ 5 – Excellent proposal, significant contribution to the state-of-the-art, good approach likely to produce expected results (top 15%)
- ☐ 4 – Good proposal, technically sound with potential contribution to the state-of-the-art (minimum passing grade)
- ☐ 3 – Adequate proposal, sound in approach and concept, likely to produce some results, but lacking in outstanding aspects, originality, or significant advancements
- ☐ 2 – Ideas not original and/or little value of the proposed work and/or lack of qualified personnel and/or inadequate facilities for the proposed work
- ☐ 1 – Fundamentally flawed idea, approach, or assumptions (please be specific in comments below)

Please provide your assessment of the following (use additional pages as necessary):

1. Technical merits of the proposed research and development

The work is technically feasible.

The research described has two key objectives each with several sub-tasks that build on the prior work for AFSOR. The underlying science behind this proposal is based on understanding the underlying mechanisms for the remarkable survival of Deinococcus radiodurans cells to ionizing radiation and desiccation at levels that cause extensive DNA damage, and the more recent evidence that led to the hypothesis that the protective mechanisms is based on small-molecule Mn^{2+} antioxidants that preserve the high efficiency of DNA repair and replication proteins during irradiation. It has been demonstrated that the combination of Mn^{2+} and small molecules such as peptides and orthophosphate (=Pi = inorganic phosphate) do in fact form catalytic Reactive oxygen species (ROS)-scavenging complexes which specifically protect proteins. Additionally, Mn^{2+} complexes reconstituted in the laboratory are extremely radioprotective in vitro and in vivo, far beyond the levels of protection conferred by conventional antioxidants. Several bacterial models from various laboratories have demonstrated that the antiquated idea that DNA double-strand breaks (DSBs) were the universal critical lesions in irradiated cells is false. One of the key findings relevant to the protection of human DNA is the fact that Mn^{2+} -peptide complexes are most protective if they contain histidine and the fact that D. radiodurans c confer their ability to survive extreme cellular insults caused by ionizing radiation onto cultured human cells.

The technical plan is to employ mass spectrometry (MS) followed by Electron Spin Echo and Electron paramagnetic resonance (ESE-EPR) along with Electron nuclear double resonance (ENMR) and Electron Spin Echo Envelope Modulation (ESEEM) to size-fractionated D. radiodurans cell extracts (and extracts from other bacteria and yeast) in order to probe the

*coordination sphere of Mn^{+2} in radiation resistant organisms such as *D. radiodurans*. The purpose is to identify the major LMW representatives and determine the Mn^{+2} coordination within the samples. ESE-EPR is useful for the detection and identification of free radicals and paramagnetic centers and can definitely be employed for the proposed studies.*

Their plan to apply ESE-EPR and the advanced paramagnetic resonance techniques of ENDOR and ESEEM spectroscopies to probe the coordination sphere of Mn^{2+} in extremely radiation-resistant prokaryote cells and yeast, and for Mn^{2+} complexes reconstituted in vitro to determine where Mn is bound; and to apply newly-developed quantitative radiochemical approaches to characterize the mechanisms of ROS-scavenging by the reconstituted Mn^{2+} complexes.

It is entirely reasonable and technically feasible to utilize these approaches to enable us to better understand the structural and functional nature of prokaryotic Mn^{2+} complexes.

2. Potential relationship of the proposed research and development to the Department of Defense

The application of the knowledge gained from these experiments is potentially tremendous. The understanding of these systems is likely to will allow us to harness the protective functions devised by these microorganisms for the practical purpose of products that will provide us with radiation protection and/or recovery from radiation damage.

Manganous metabolites are increasingly evident in all branches of life. It is an essential transition metal that, among other functions, can act independently of proteins to either defend against or promote oxidative stress and disease. When there is an orthophosphate complex of Mn^{2+} it can act to produce resistance to oxidative stress There is clear evidence demonstrating that DNA repair proteins can, when intact, repair hundreds or thousands of radiation-induced DSBs, so long as they are not inactivated by oxidation by reactive oxygen species (ROS). Thus the presence of these complexes can allow for that DNA repair.

There is a realistic probability that Mn^{2+} -peptide complexes from these lower organisms can be adapted for the use of protecting citizens and troops from radiation damage.

In other words, because these insights have a very high probability of facilitating approaches to protect cells and biomaterials from radiation damage, it is appropriate that AFOSR continues to support research into the mechanisms extremophiles use to protect their proteins.

3. The likelihood of the proposed effort to develop new research capabilities and broaden the research base in support of U.S. national defense

Among the many deadly weapons and devices that can be brought to bear against our citizens and our troops are nuclear and radiation weapons. A very significant part of that problem stems not from the blast injuries but from the radiation damage. Radiation injury can be a hazard from accidents at nuclear plants and even from medicinal use of radiation. The effort described here brings us closer to a solution for radiation damage to ourselves, our animals, plants, and biomaterials. It is self-evident a project that has the potential and great likelihood of developing a realistic solution to radiation damage is vital to our national security.

4. The proposer's, principal investigator's, team leader's, or key personnel's qualifications, capabilities, related experience, facilities, or techniques or a combination of these factors that are integral to achieving USAF objectives

*Dr. Michael Daly is an accomplished scientist. He has been Professor of Pathology and Molecular & Cell Biology since 2007, served on the Committee on Outer Planets for the National Academy of Sciences 2010-2012 and is the chairman, USU Radiation Safety Committee -a role he has held since 2005. He has extensive experience working in the laboratory with prokaryotic organisms – in particular various *Deinococcus* species and with fungi. He has published extensively in the most prestigious peer-reviewed journals internationally and nationally and presented his work at major national and international conferences to great acclaim. He has also been featured in films and other media highlighting his important scientific work.*

Dr. Daly's lab has the equipment needed for various aspects of this project. For example his lab has the equipment for the sample preparation prior to ENDOR (cells and reconstituted Mn^{2+} complexes).

The materials, equipment and infrastructure, including ^{60}Co and ^{137}Cs radiation facilities at USU (Laboratory Resources), necessary to conduct the primary research are state-of-the-art, well-maintained, and secure. Specifically, Dr. Daly is licensed for use of three DoD irradiators, the labs at USU include high sensitivity, high throughput Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) and an Applied Biosystems Voyager-DE STR BioSpectrometry Workstation, a robotic microarray pin spotter from Cartesian (model PixSys 5500); the ScanArray® 5000 Microarray Analysis System (GSI Lumonics), equipped with 4 lasers, Gas chromatography/mass spectrometry and HPLC Exclusion Chromatography equipment at USU are also state-of-the-art.

*Dr. Brian Hoffman has the equipment needed for ESE-EPR/ENDOR/ESEEM spectroscopy studies on in vivo and in vitro Mn complexes of *D. radiodurans* and other extremely radiation-resistant extremophiles at the Department of Chemistry, Northwestern University, Evanston, IL. At this facility he has 35 GHz Varian E-109 CW EPR/ENDOR Spectrometer, a Pulsed X-band EPR/ENDOR Spectrometer, Pulsed Q(Ka)-Band EPR/ENDOR Spectrometer, and a W-Band EPR/ENDOR spectrometer. This equipment is also state-of-the-art.*

5. The proposer's and associated personnel's record of past performance

*Dr. Michael Daly has been working on radiation resistance since the 1990s and is highly regarded nationally and internationally in this field. Dr. Daly and his team have made many dramatic contributions that have led to paradigm-shifting view of radiation toxicity and paved the way to for the exploration of new approaches to cellular radioprotection. Dr. Daly has been studying the role of antioxidant Mn^{2+} -metabolite complexes in the extremely radiation-resistant bacterium *Deinococcus radiodurans* since 2003.*

*Dr. Daly is expert in the use of EPR to characterize the Mn^{2+} content of *D. radiodurans* cell extracts and has had several publications on this topic in major journals such as his paper in Science in 2004. He has applied similar techniques to evaluate radiation damage and DNA repair in a number of other organisms from bacteria to fungi.*

Dr. Brian Hoffman (Member, US National Academy of Sciences) at Northwestern University is a long-time collaborator of Dr. Daly. Dr. Hoffman is renowned for his studies involving electron transfer and in the use of electron paramagnetic resonance (EPR) spectroscopy and the advanced paramagnetic resonance techniques of electron nuclear double resonance (ENDOR) and electron spin echo envelope modulation (ESEEM) spectroscopies. He introduced the use of ENDOR spectroscopy to study the in vivo coordination and speciation of Mn^{2+} in cells.

6. The realism and reasonableness of proposed costs

The costs for salary for the personnel is in-keeping with current salaries for this work and very reasonable. The researchers are asking only for domestic travel costs and the sum requested is minimal. The cost requested for equipment and supplies is less than expected considering the high level of sophistication of the needed equipment and the nature of the research. The total sum requested is highly appropriate for the work and a very reasonable expense for a project with a very high likelihood of yielding practical solutions for a serious national security issue.

Recommendation:

- ☒ Accept
☐ Decline
☐ Other (provide comments)

Comments:

I am highly impressed with the proposed research and unequivocally endorse this proposal.

4.7 Progress towards Original Research Goals: Publications (2009-2013) under AFOSR Award: FA9550-10-1-0118

1. Ajay Sharma, Elena K. Gaidamakova, Vera Y. Matrosova, Brian Bennett, **MICHAEL J. DALY**, Brian M. Hoffman (2013) Responses of Mn²⁺ speciation in *Deinococcus radiodurans* and *Escherichia coli* to γ -radiation by advanced paramagnetic resonance methods. *Proc. Natl. Acad. Sci. USA* 110(15):5945-50.

Abstract: The remarkable ability of bacterium *Deinococcus radiodurans* to survive extreme doses of γ -rays (12,000 Gy), 20 times greater than *Escherichia coli*, is undiminished by loss of Mn-dependent superoxide dismutase (SodA). *D. radiodurans* radiation resistance is attributed to the accumulation of low-molecular-weight (LMW) "antioxidant" Mn(2+)-metabolite complexes that protect essential enzymes from oxidative damage. However, in vivo information about such complexes within *D. radiodurans* cells is lacking, and the idea that they can supplant reactive-oxygen-species (ROS)-scavenging enzymes remains controversial. In this report, measurements by advanced paramagnetic resonance techniques [electron-spin-echo (ESE)-EPR/electron nuclear double resonance/ESE envelope modulation (ESEEM)] reveal differential details of the in vivo Mn(2+) speciation in *D. radiodurans* and *E. coli* cells and their responses to 10 kGy γ -irradiation. The Mn(2+) of *D. radiodurans* exists predominantly as LMW complexes with nitrogenous metabolites and orthophosphate, with negligible EPR signal from Mn(2+) of SodA. Thus, the extreme radiation resistance of *D. radiodurans* cells cannot be attributed to SodA. Correspondingly, 10 kGy irradiation causes no change in *D. radiodurans* Mn(2+) speciation, despite the paucity of holo-SodA. In contrast, the EPR signal of *E. coli* is dominated by signals from low-symmetry enzyme sites such as that of SodA, with a minority pool of LMW Mn(2+) complexes that show negligible coordination by nitrogenous metabolites. Nonetheless, irradiation of *E. coli* majorly changes LMW Mn(2+) speciation, with extensive binding of nitrogenous ligands created by irradiation. We infer that *E. coli* is highly susceptible to radiation-induced ROS because it lacks an adequate supply of LMW Mn antioxidants.

2. Valeria C. Culotta, **MICHAEL J. DALY** (2013) Manganese Complexes: Diverse Metabolic Routes to Oxidative Stress Resistance in Prokaryotes and Yeast. *Antioxidants & Redox Signaling* (Accepted, doi: 10.1089/ars.2012.5093).

Abstract: Antioxidant enzymes are thought to provide critical protection to cells against reactive oxygen species (ROS). However, many organisms can fully compensate for the loss of such enzymatic defenses by accumulating metabolites and Mn(2+), which can form catalytic Mn-antioxidants. Accumulated metabolites can direct reactivity of Mn(2+) with superoxide and specifically shield proteins from oxidative damage. Recent Advances: There is mounting evidence that Mn-Pi (orthophosphate) complexes act as potent scavengers of superoxide in all three branches of life. Moreover, it is evident that Mn(2+) in complexes with carbonates, peptides, nucleosides, and organic acids can also form catalytic Mn-antioxidants, pointing to diverse metabolic routes to oxidative stress resistance. Critical Issues: What conditions favor utility of Mn-metabolites versus enzymatic means for removing ROS? Mn(2+)-metabolite defenses are critical for preserving the activity of repair enzymes in *Deinococcus radiodurans* exposed to intense radiation stress, and in *Lactobacillus plantarum*, which lacks antioxidant enzymes. In other microorganisms, Mn-antioxidants can serve as an auxiliary protection when enzymatic antioxidants are insufficient or fail. These findings of a critical role of Mn-antioxidants in the survival of prokaryotes under oxidative stress parallel the trends developing for the simple eukaryote *Saccharomyces cerevisiae*. Future Directions: Phosphates, peptides and organic acids are just a snapshot of the types of anionic metabolites that promote such reactivity of Mn(2+). Their probable roles in pathogen defense against the host immune response and in ROS-mediated signaling pathways are also areas that are worthy of serious investigation. Moreover, it is clear that these protective chemical processes can be harnessed for practical purposes.

3. L.A. Klobutcher, E.K. Gaidamakova, **MICHAEL J. DALY**, B. Setlow, P. Setlow (2013) Effects of Mn²⁺ levels on the resistance properties of *Bacillus cereus* spores. *Journal of Bacteriology Research*, 5(1):9-12.

Abstract: In some *Bacillus* species, manganese levels influence the resistance properties of spores. To determine if this was true for *Bacillus cereus*, bacteria were sporulated with different MnCl₂ concentrations resulting in spores with 30-fold differences in core Mn²⁺ levels. Spores with different Mn²⁺ levels displayed no differences in resistance to dry heat, UV radiation, γ -radiation, or hydrogen peroxide. However, spores with the lowest Mn²⁺ level were less resistant to wet heat. Overall, Mn²⁺ levels were not a major factor in *B. cereus* spore resistance, and this suggests that this will also be true for the closely related *B. anthracis* spores.

4. E.K. Gaidamakova, I.A. Myles, D.P. McDaniel, C.J. Fowler, P.A. Valdez, S. Naik, M. Gayen, P. Gupta, A. Sharma, P.J. Glass, R.K. Maheshwari, S.K. Datta, **MICHAEL J. DALY** (2012) Preserving Immunogenicity of Lethally Irradiated Viral and Bacterial Vaccine Epitopes Using a Radio- Protective Mn(2+)-Peptide Complex from *Deinococcus*. *Cell Host Microbe*, 12(1):117-124.

Abstract: Although pathogen inactivation by γ -radiation is an attractive approach for whole-organism vaccine development, radiation doses required to ensure sterility also destroy immunogenic protein epitopes needed to mount protective immune responses. We demonstrate the use of a reconstituted manganous peptide complex from the radiation-resistant bacterium *Deinococcus radiodurans* to protect protein epitopes from radiation-induced damage and uncouple it from genome damage and organism killing. The Mn(2+) complex preserved antigenic structures in aqueous preparations of bacteriophage lambda, Venezuelan equine encephalitis virus, and *Staphylococcus aureus* during supralethal irradiation (25-40 kGy). An irradiated vaccine elicited both antibody and Th17 responses, and induced B and T cell-dependent protection against methicillin-resistant *S. aureus* (MRSA) in mice. Structural integrity of viruses and bacteria are shown to be preserved at radiation doses far above those which abolish infectivity. This approach could expedite vaccine production for emerging and established pathogens for which no protective vaccines exist.

5. **MICHAEL J. DALY** (2011) Death by protein damage in irradiated cells. *DNA Repair* 11(1):12-21.

Abstract: A founding concept of radiobiology that deals with X-rays, γ -rays and ultraviolet light is that radiation indiscriminately damages cellular macromolecules. Mounting experimental evidence does not fit into this theoretical framework. Whereas DNA lesion-yields in cells exposed to a given dose and type of radiation appear to be fixed, protein lesion-yields are highly variable. Extremely radiation resistant bacteria such as *Deinococcus radiodurans* have evolved extraordinarily efficient antioxidant chemical defenses which specifically protect proteins and the functions they catalyze. In diverse prokaryotes, the lethal effects of radiation appear to be governed by oxidative protein damage, which inactivates enzymes including those needed to repair and replicate DNA. These findings offer fresh insight into the molecular mechanisms of radiation resistance and present themselves as new opportunities to study and control oxidative stress in eukaryotes, including mammalian cells and their cancer cell counterparts.

6. **MICHAEL J. DALY** (2011) The Achilles' heel of radiation resistant prokaryotes. In *Assessment of Planetary Protection Requirements for Spacecraft Missions to Icy Solar System Bodies* (Sogin ML et al., eds). Division on Engineering and Physical Sciences, National Research Council of the National Academy of Sciences.

7. S. Ghosh, A. Ramirez-Peralta, E. Gaidamakova, P. Zhang, Y.Q. Li, **MICHAEL J. DALY**, P. Setlow (2011) Effects of Mn levels on resistance of *Bacillus megaterium* spores to heat, radiation and hydrogen peroxide. *J Appl Microbiol.*, 111(3):663-670.

8. A.C. Granger, E.K. Gaidamakova, V.Y. Matrosova, **MICHAEL J. DALY**, P. Setlow (2011) Effects of Mn and Fe levels on *Bacillus subtilis* spore resistance and effects of Mn²⁺, other divalent cations, orthophosphate, and dipicolinic acid on protein resistance to ionizing radiation. *Appl Environ Microbiol.*, 77(1):32-40.

9. **MICHAEL J. DALY**, E.K. Gaidamakova, V. Y. Matrosova, J.G. Kiang, R. Fukumoto, D.Y. Lee, N.B. Wehr, G.A. Viteri, B.S. Berlett, R.L. Levine (2010) Small-molecule antioxidant proteome-shields in *Deinococcus radiodurans*. *PLoS ONE*, 5(9), e12570.

10. K. S. Makarova, **MICHAEL J. DALY** (2010) Comparative genomics of stress response systems in *Deinococcus* bacteria. Storz G, Henne R (Eds) In *Bacterial Stress Responses*.

11. **MICHAEL J. DALY** (2010) *Deinococcus radiodurans*: revising the molecular basis for radiation effects on cells. *Extremophiles Handbook*. Horikoshi, K. (Ed.). Springer, Japan.

12. **MICHAEL J. DALY** (2009) *Deinococcus* prospects. *Nat. Rev. Microbiol.*, 7, 476.

13. **MICHAEL J. DALY** (2009) A new perspective on radiation resistance based on *Deinococcus radiodurans*. *Nat. Rev. Microbiol.*, 7, 237-245.

5. Personnel Supported: List professional personnel (Faculty, Post-Docs, Graduate Students, etc.) supported by and/or associated with the research effort.

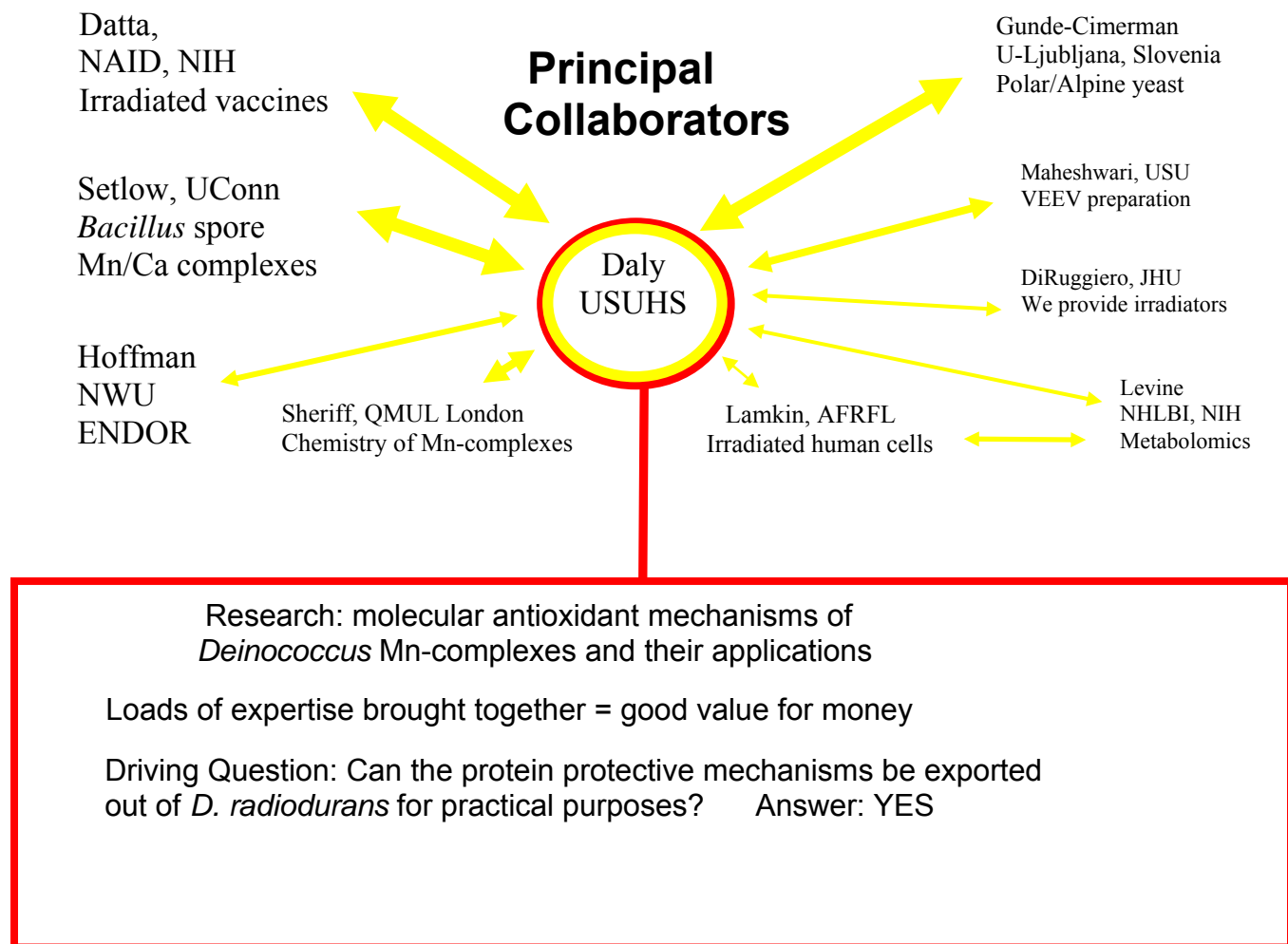
Personnel: 1 PI + 1.5 Research Associates, and 1 PhD Student funded by the AFOSR grant:

PI: M. J. Daly, Ph.D., Professor (no salary from AFOSR grant)
Senior Res. Associate: Dr. Elena Gaidamakova, Ph.D. (100% salary from AFOSR grant)
Senior Res. Associate: Dr. Vera Matrosova, Ph.D. (50% salary from AFOSR grant)
1 Ph.D. Graduate Student (Maggie Wear @ USUHS) (no salary from AFOSR grant)

6. 2012-2013 Peer-Reviewed Publications

1. Ajay Sharma, Elena K. Gaidamakova, Vera Y. Matrosova, Brian Bennett, **MICHAEL J. DALY**, Brian M. Hoffman (2013) Responses of Mn²⁺ speciation in *Deinococcus radiodurans* and *Escherichia coli* to γ -radiation by advanced paramagnetic resonance methods. *Proc. Natl. Acad. Sci. USA* 110(15):5945-50.
2. Valeria C. Culotta, **MICHAEL J. DALY** (2013) Manganese Complexes: Diverse Metabolic Routes to Oxidative Stress Resistance in Prokaryotes and Yeast. *Antioxidants & Redox Signaling* (doi: 10.1089/ars.2012.5093).
3. Lawrence A. Klobutcher, Elena Gaidamakova, **MICHAEL J. DALY**, Barbara Setlow, Peter Setlow (2013) Effects of Mn²⁺ levels on the resistance properties of *Bacillus cereus* spores. *Journal of Bacteriology Research* 5(1):9-12.
4. E.K. Gaidamakova, I.A. Myles, D.P. McDaniel, C.J. Fowler, P.A. Valdez, S. Naik, M. Gayen, P. Gupta, A. Sharma, P.J. Glass, R.K. Maheshwari, S.K. Datta, **MICHAEL J. DALY** (2012) Preserving Immunogenicity of Lethally Irradiated Viral and Bacterial Vaccine Epitopes Using a Radio- Protective Mn(2+)-Peptide Complex from *Deinococcus*. *Cell Host & Microbe*, 12(1):117-124.

7. Most Recent Interactions/Transitions



• Sandip Datta –

<http://www.niaid.nih.gov/LabsAndResources/labs/aboutlabs/lcid/bacpathogenesissection/pages/sandipdatta.aspx>

Tests the protective efficacy of Mn-DP-Pi-irradiated bacterial vaccines.

• Peter Setlow – http://grad.uchc.edu/faculty/bios_old/setlow.html

Prepares *Bacillus* spores with varying Mn contents and studies effect on stress resistance.

• Brian Hofmann – <http://chemgroups.northwestern.edu/hoffman/>

Conducts ENDOR of *D. radiodurans*, *E. coli* and *S. cerevisiae*, and Mn complexes.

• Tippu Sheriff – <http://www.sbcs.qmul.ac.uk/staff/tippusheriff.html>

Performs EPR-based structural analyses on reconstituted *Deinococcus* complexes.

• Thomas Lamkin – AFRFL – (Funded separately by AFOSR)

Tests radioprotective properties of reconstituted *Deinococcus* Mn complexes

• Rod Levine –

https://intramural.nhlbi.nih.gov/labs/LB_G/LBLL/Pages/ResearchInfo.aspx

Studies sequence dependent radioprotective properties of *Deinococcus* complexes.

• Jocelyne DiRuggiero – <http://www.bio.jhu.edu/Faculty/DiRuggiero/>

Studies small-molecule antioxidants in archaea.

• Radha Maheshwari – <http://www.usuhs.mil/pat/rmaheshwari.html>

Tests the protective efficacy of Mn-DP-Pi-irradiated viral vaccines.

• Nina Gunde-Cimerman – <http://www.fkkt.uni-lj.si/en/?468>

Provides alpine yeast for studies on radiation-induced protein oxidation.

- Negotiations underway with serial venture capitalists engaged in start-up for ‘Irradiated-Vaccine Company’, New York, NY; Jonathan Gottlieb, PhD, MBA. Based on Daly Patent: ‘Applications of Manganese-Peptide Complexes towards the Preparation of Irradiated Vaccines.’ (2012) Morgan, Lewis & Bockius LLP, 1111 Pennsylvania Avenue, NW | Washington, DC 20004.

- HIV/SIV for proof-of-concept studies for Mn-complex irradiated vaccines. Negotiations with Merlin Rob, MD, HJF Director of Clinical Research for the US Military HIV Research Program.

- Expanding applications of EPR/ENDOR/ESEEM Spectroscopies towards quantifying cellular radiation resistance for medical applications: Responses of Mn^{2+} Complexes in *Deinococcus radiodurans*, *Escherichia coli*, and *Saccharomyces cerevisiae* to Ionizing Radiation.

- New approaches to controlling the resistance properties of *Bacillus* spores: Effects of Mn^{2+} Levels on the Resistance Properties of *Bacillus cereus* Spores, with Peter Setlow.

2012-2013 Research Transitions

Daly/LLNL: Bioremediation of acidic radioactive waste environments

Daly/DTRA: Irradiated Vaccine against Venezuelan Equine Encephalitis Virus

NIH (NIAID): MRSA vaccine development, with Sandip Datta, MD. Chief, Bacterial Pathogenesis

8. Provisional Patents under Grant FA9550-10-1-0118

HMJAMM HAS SPONSORED THE FOLLOWING PATENT APPLICATIONS ON BEHALF OF DALY:

1) COMPOSITIONS CONTAINING PURINE NUCLEOSIDES AND MANGANESE AND THEIR USES.

2) COMPOSITIONS CONTAINING PURINE AND PYRIMIDINE NUCLEOSIDES, PEPTIDES, AND MANGANESE AND THEIR USES.

3) PRESERVING IMMUNOGENICITY OF LETHALLY IRRADIATED VIRAL AND BACTERIAL VACCINE EPITOPES USING A RADIO-PROTECTIVE Mn^{2+} -PEPTIDE COMPLEX FROM DEINOCOCCUS RADIODURANS. DEVELOPMENT OF A VACCINE AGAINST MRSA.

MORGAN, LEWIS & BOCKIUS LLP, 1111 PENNSYLVANIA AVENUE, NW | WASHINGTON, DC 20004. TODD B. BUCK, PH.D., J.D., DIRECT: 202.739.5698 | MAIN: 202.739.3000 | TBUCK@MORGANLEWIS.COM | HMJFAMM REP: AIDA CREMESTI, PHD. 240.694.2208. HJF-165-3 PROV 3.

9. 2012-2013 Honors/Awards:

‘List honors and awards received during the grant/contract period.’

Inter-governmental (US/Slovenia) science travel award: For Drs. Daly and Gunde-Cimerman - Covers reciprocal air travel and accommodation (six weeks) between Slovenia and Washington, DC, to foster collaborative research on: Mechanisms of Extreme Oxidative Stress Resistance in Yeast and Fungi of the Julian Alps.